8-1-2018

The Effects of Fluopyram on Macrophomina phaseolina and Charcoal rot of Soybean

Annie Caroline Padgett
Southern Illinois University Carbondale, akorando@siu.edu

THE EFFECTS OF FLUOPYRAM ON MACROPHOMINA PHASEOLINA AND CHARCOAL ROT OF SOYBEAN

by

Annie C. Padgett

B.S., Southern Illinois University, 2015

A Thesis
Submitted in Partial Fulfillment of the Requirements for the
Master of Science Degree

Department of Plant, Soil, and Agricultural Systems
in the Graduate School
Southern Illinois University Carbondale
August 2018

THESIS APPROVAL

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Approved by:

Dr. Jason Bond

Dr. Ahmad Fakhoury

Dr. Seburn Pense

Graduate School
Southern Illinois University Carbondale
April 6, 2018

AN ABSTRACT OF THE THESIS OF

Annie C. Padgett, for the Master of Science degree in PLANT, SOIL, and AGRICULTURAL SYSTEMS, presented on April 6, 2018, at Southern Illinois University Carbondale.

TITLE: THE EFFECTS OF FLUOPYRAM ON *MACROPHOMINA PHASEOLINA* AND CHARCOAL ROT OF SOYBEAN

MAJOR PROFESSOR: Dr. Jason Bond

Macrophomina phaseolina (Tassi) Goid., is a soilborne fungus that causes charcoal rot in over 500 plant species including *Zea mays* L. and *Glycine max* (L.) Merr. The pathogen is present in most soybean growing regions of the United States. Infection in soybean can occur after emergence and throughout the vegetative growth stages in a range of environmental conditions. The syndrome is manifest during periods of hot and dry conditions during the reproductive states of the crop. Management options are lacking and consist of avoidance and irrigating crops to lessen the damage caused by the pathogen. Fluopyram is a succinate dehydrogenase inhibitor (SDHI) fungicide with a spectrum of activity against a unique and very diverse group of plant pathogens including species of *Venturia, Botrytis*, *Alternaria*, *Sclerotina*, *Monilia* and multiple species that cause powdery mildew. This fungicide also has activity against *Fusarium virguliforme* O'Donnell & T. Aoki, *Heterodera glycines* Ichinohe (soybean cyst nematode), *Meloidogyne incognita* acrita (root knot nematode) and other important nematode species. The objective of this research was to determine the impact of fluopyram on colonization by *M. phaseolina* and symptoms of charcoal rot and on plant emergence, plant height and soybean yield. A field study was initiated at the SIU Agronomy Research Station in Carbondale in 2015 and 2016. A factorial treatment structure was used with variety and fungicide treatment as the two factors. Four soybean varieties of varying maturity and three different seed treatment options. The first treatment contained a base fungicide, insecticide and a nematode biocontrol

agent. The second treatment contained the base fungicide, insecticide, nematode biocontrol agent and fluopyram. The third treatment was a non-treated control. The four varieties and 3 seed treatment options were used in all possible combinations with five replications in a randomized complete block design. Each four-row plot was 3.04 m wide by 6.1 m in length with 0.76 m row spacing. The plots were infested at planting with *M. phaseolina* infested sorghum seed at the rate at 4.0 g of inoculum per 30.5 cm of row. Data collected included stand, plant height, seed quality and soybean yield. Soybean cyst nematode (SCN) samples were collected 2 weeks after planting. Root samples and root ratings were collected at 1 month after emergence and prior to harvest to determine colony forming units and for DNA extraction to quantify *M. phaseolina* in the roots using qPCR. For both growing seasons, 2015 and 2016 there was more rainfall than the 29-year average. The average air temperature was consistent with the 26-year average. There was varietal differences in qPCR for both 30 days after planting (DAP) and 120 DAP. For seed treatment options, there were not differences between the treatments for the 30 DAP, but 120 DAP showed differences. For CFU there was varietal differences but no differences between seed treatments. There were no differences between seed treatments for plant height. Seed quality was assessed in 2015. There were differences across varieties but not across seed treatment. Soybean yield differed among varieties but not seed treatment.

ACKNOWLEDGMENTS

I would like to take the opportunity and thank everyone that has helped me the last few years to pursue my master's degree. Thank you to all the student workers who spent many hours digging roots, splitting roots, plating and counting CFU. I greatly appreciated all the hard work and time you guys put into my research. I want to thank Dr. Fakhoury's lab, especially Arjun Subedi for teaching me the molecular side of things and letting me use your lab for long periods of time.

To my major professor, Dr. Jason Bond, thank you for believing in me and offering the encouragement I needed to pursue a master's degree. The advice and guidance you offered were invaluable in helping me achieve my goal. Thank you Dr. Ahmad Fakhoury and Dr. Seb Pense for taking the time and serving on my committee. To the professors of Crops, Soil and Environmental Management, thank you for everything you have taught me throughout the years. Coming to SIU and not knowing what I wanted to pursue quickly changed because of the classes. I want to thank Chris Vick and Amelia Vick for everything you guys have taught me in the field, lab and at the greenhouse.

Most importantly, I want to thank my family. Saying thank you isn't enough for all the love and support you guys have provided me throughout this journey. Without you all, I would not be where I am at today. Thank you for guiding me in the right direction and teaching me at a young age to work hard and never give up. It has shaped me into the person I am today.

To my husband Jordan, thank you for being by my side through it all and never letting me throw my hands up when times were rough. Thank you for all the countless pep talks.

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CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

Soybeans [*Glycine max* (L.) Merr.] serve as one of the most valuable crops in the world, not only as an oil seed crop and feed for livestock and aquaculture, but as a good source of protein for the human diet and as a biofuel feedstock (Masuda et al, 2009). Soybeans are the dominant oilseed in the United States, accounting for about 90% of United States oilseed production and is the second most planted field crop in the United States after corn (USDA, 2017). The legume crop has the highest protein content and the highest gross output of vegetable oil among the cultivated crops in the world (Qiu et al., 2010). Soybean originated in China and for the first half of the 20th century China was the largest producer and exporter. In the 1950s soybean production developed rapidly in the United States (Qiu et al., 2010). Now it is the largest soybean producing country in the world (Guriqbal, 2010). The first documentation of soybean usage in the United States was in 1765 in the state of Georgia (Hartman et al., 2011). At that time soybeans were grown and processed to make products for export, such as margarine or shortening (Hartman et al, 2011). The discovery in 1917 that heating soybean meal made it suitable as livestock feed, led to the growth of the soybean processing industry and the dual-purpose protein and oil crop of today (Hartman et al., 2011). By the 1970s the United States expanded its production and supplied two thirds of the world's soybean needs (Hartman et al., 2011). Soybean production in 2015 totaled a record 3.93 billion bushels with the average yield per acre estimated at a record high 48.0 bushels and total harvested area being 81.8 million acres (USDA, 2016).

Charcoal rot of soybean caused by the pathogen *Macrophomina phaseolina* (Tassi) Goid. is one of the most important diseases of soybean (Almeida et al., 2008). *M. phaseolina* is known

to cause disease in at least 500 plant species including economic hosts such as corn, sorghum, cotton, and tobacco (Mengistu et al., 2009). The pathogen is widely present in soil and has been reported to cause yield losses of 30 to 50%, mainly in southern soybean production regions of the United States (Yang et al., 2005). Although, the disease can occur throughout the north central and southern regions of the United States, as well as in tropical and subtropical regions of the world (Wyllie, 1988). Charcoal rot in soybean was first observed in the United States in 1949 (Young, 1949). The disease has emerged, however, from being minor to more severe within the last few years.

Colonization of host tissue is favored by high temperature and low soil moisture (Dhingra and Sinclair, 1974). Low soil moisture has been reported to increase growth (Mulrooney, 1988) and enhance survival (Short et al., 1980) of the pathogen. Drought conditions favor the development of charcoal rot in sorghum and sunflower, and yield suppression has been attributed to the influence of the combination of drought and charcoal rot (Gary et al., 1991; Manici et al., 1995). Specifically, severity of charcoal rot has been determined to be significant when air and soil temperatures are high (28 to 35°C), and when soil moisture is limiting (Gary et al., 1991; Pearson et al., 1984; Smith and Wyllie, 1999). Damage caused by *M. phaseolina* can increase under either type of stress separately, as well as in combination (Hartman et al., 1999). The fungus has both a broad host range and wide geographic distribution (Grau et al., 2004). The fungus survives in the soil and on soybean debris as microsclerotia; which are black, spherical to oblong, and typically measure 50 to 200 µm in diameter (Smith and Wyllie, 1999). After harvest of the infected crop, the microsclerotia are protected in the crop residues and are then released into the soil after crop residues break down (Gupta et al., 2012). Microsclerotia can survive in the soil for 2-15 years depending on environmental conditions and whether or not the sclerotia

are associated with host residue (Cook et al., 1973; Papavizas, 1977; Short et al., 1980). Microsclerotia produced in the roots and stem tissues of its hosts serve as the primary source of inoculum (Kaur et al., 2012). Microsclerotia can germinate on the surface of or close to the roots and germination can occur throughout the growing season as long environmental conditions remain favorable (Wyllie, 1988). Infected soybean seed can also be a source of inoculum (Smith et al., 2015).

Root infection can occur throughout the entire development of the soybean life cycle. Invasion of the root cortex is followed by colonization of vascular tissue (Grau et al., 2004). Mycelia colonize the vascular tissue by growing through the cortex and then entering the xylem vessels (Abawi and Pastor-Corrales, 1990). Once inside vascular tissue, the fungus spreads through the tap root and plugs the vessels resulting in wilting of the plant (Wyllie, 1988). The fungus will then grow outward throughout the root and stem tissue and eventually produces visible microsclerotia later in the growing season (Wyllie, 1988). Once the soybean plant reaches the reproductive stages, the fungus starts to grow rapidly and disturbs water uptake by clogging vascular tissue with fungal growth and newly formed microsclerotia (Smith et al., 2015). When numerous microsclerotia are present it will give the lower stem and taproot tissue a charcoal-like appearance that provides inoculum for future disease (Smith et al., 2015).

Symptoms of charcoal rot can be found at all stages of the soybean plant. Infected seeds are either asymptomatic or have microsclerotia, appearing as black spots of variable size, embedded in cracks of the seed coat (Gangopadhyay et al., 1970). The infected seed may germinate but resultant seedlings usually die within a few days (Kunwar et al., 1986). The pathogen causes lesions on the roots, stems, pods and seeds (Gupta et al., 2012). Above ground symptoms generally appear after flowering; particularly at R5, R6 and R7 growth stages (Fehr et

al., 1971). Diseased plants initially show non-specific symptoms such as reduced leaf size and stem height, which indicate loss of vigor (Grau et al., 2004). At flowering, a light gray discoloration develops on the epidermal tissues of both the tap and secondary roots and lower stems (Grau et al., 2004). Microsclerotia are formed in the vascular tissues and in the pith, giving a greyish-black appearance to the subepidermal tissues of the stem (Gupta et al., 2012). Leaves of infected plants remain smaller than normal and subsequently turn yellow prior to wilting (Gupta and Chauhan, 2005). The infected mature and dry pods are covered with locally or widely distributed microsclerotia. The infected crop exhibits premature yellowing in scattered patches (Gupta et al., 2012). *M. phaseolina* can reduce plant height, root volume and root weight by more than 50% which can contribute to yield loss (Wyllie, 1976). Ammon et al. (1974, 1975) used scanning electron microscopy to study the early stages of fungal infection. In both soybean and *Medicago truncatula, in-vitro* infection and microscopic examination of infected roots revealed that the initial host entry occurred within 24 h, followed by a rapid colonization 36-48 h after inoculation (Bressano et al., 2010; Gaige et al., 2010).

Interaction between charcoal rot and soybean cyst nematode (SCN) has been documented from early reports (Todd et al., 1987; Meyer et al., 1974) and suggests that *H. glycines* infection can increase colonization of soybean roots by *M. phaseolina* and may increase losses due to charcoal rot (Radwan et al., 2014). Severity of charcoal rot, which is frequently related to stress (Meyer et al., 1974; Pearson et al., 1984), may be enhanced subsequently in the presence of the nematode (Todd et al., 1987).

Management options are lacking for the disease but there are some practices that may help reduce the impact of the disease. Most recommendations focus on reducing crop stress and maintaining plant vigor (Grau et al., 2004). Host resistance may be the only feasible method to

manage the disease (Bristow et al., 1984; Smith and Wyllie, 1999; Wyllie, 1988), but host resistance is currently not available, and other management options are needed (Mengistu et al., 2007; Mengistu et al., 2009). Planting at a lower population rate can reduce the incidence of charcoal rot as well as reduction in microsclerotia (Gupta et al., 2012). Crop rotation with non-host crop, or a crop that is less-susceptible to *M. phaseolina* for a sufficient time, reduces infection (Grau et al., 2004). Rotation with non-host crops for 2-3 years is necessary to lower *M. phaseolina* infection levels in severely infested fields (Gupta et al., 2012). Cotton in rotation with soybean consistently reduced the population density of *M. phaseolina* more than did corn-soybean rotations (Wrather et al., 1998). These results suggest that only long-term rotations of soybean with cotton or corn would be effective in decreasing the population density of *M. phaseolina* to non-damaging levels (Wrather et al., 1998). Irrigation is recommended to help alleviate stress; although, Kendig et al., (2000) showed that water management limits, but does not prevent root infection by *M. phaseolina*. An alternative method to reduce the pathogen population density is through tillage (Wrather et al., 1998). Wyllie (1988) reports that there is little direct evidence on the role of plant nutrition or soil fertility on the disease. Limited information is available on the direct role of fertility or plant nutrition in charcoal rot management in soybean (Todd et al., 1987). Recent studies indicate that an increase in nitrogen:phosphorus:potassium (NPK) supply (Csöndes et al., 2008) is important for charcoal rot management in soybeans.

Problem Statement

Current seed treatments have been reported to have limited impact on Charcoal rot severity in field trials (Mengistu et al., 2015). Currently, no chemicals are available to control charcoal rot in soybeans (Reznikov et al., 2016). In a recent study, two biological products

(*Trichoderma viride* and *Bacillus subtilis*) or one chemical treatment (thiophanate methyl + pyraclostrobin) reduced disease severity in field trials (Reznikov et al., 2016). Recent *in-vitro* research has revealed that fluopyram has a negative impact on *M. phaseolina* (A. Fakhoury, personal communication, April 1, 2015), and additional trials are needed to evaluate the potential for use in production fields.

The research objectives were to:

1. Determine the impact of fluopyram on colonization by *M. phaseolina* and symptoms of charcoal rot.
2. Determine the impact of fluopyram on plant parameters such as emergence, plant height and soybean yield.

The fungicides that were evaluated included EverGol Energy (0.019 mg a/seed), Allegiance FL (0.02 mg a/seed), Poncho/Votivo (0.13 mg a/seed) and ILeVO (0.15 mg a/seed). EverGol Energy is a fungicide that targets soilborne, seedborne and early season post-emergence diseases (Anonymous, 2016). The active ingredients (ai) of EverGol Energy is Prothioconazole, a broad spectrum, systemic fungicide that belongs to the conazole (triazolinthione) class of fungicide (Kashuba et al., 2006). Conazole fungicides act through disruption of normal fungal cell membrane structure and function primarily through interactions or inhibitions of ergosterol synthesis (Kashuba et al., 2006). Penflufen is a systemic, xylem-mobile fungicide (Anonymous, 2012). The fungicide is a carboxamide fungicide that inhibits mitochondrial respiration by inhibiting succinate dehydrogenase, an enzyme in the electron transport system (Anonymous, 2012). Penflufen has fungicidal activity against many phytopathogenic fungi (such as *Rhizoctonia spp*. and *Ustilago spp*.) Metalaxyl, a systemic fungicide with protective and curative properties that controls species of *Pythium* and *Phytophthora* and targets seed rots and is

absorbed through the leaves, stems and roots (Agrochemical, 2013). Metalaxyl suppresses sporangial formation, mycelia growth and the establishment of new infections by the inhibition of RNA synthesis (Cohen et al., 1986). The active ingredient clothianidin, is a neonicotinoid insecticide. Neonicotinoids are among the most effective insecticide for the control of sucking insect pests (Elbert et al., 2008). All neonicotinoids act as agonists on the insect nicotinic acetylcholine receptor (nAChR) (Elbert et al., 2008). *Bacillus firmus* i-1582 is the active ingredient in Votivo. *Bacillus firmus* i-1582 is a bacterial strain that has nematicidal activity against plant-pathogenic nematodes (Peleg et al., 2002). Fluopyram is a systemic seed treatment used for protecting the seed and seedling against certain early season plant pathogenic fungi and nematodes that attack the root system. Fluopyram is grouped under the FRAC Code No. 7 (List, 2005) and is a succinate dehydrogenase inhibitor (SDHI) (Sierotzki et al., 2013). Fluopryam inhibits spore germination, germ tube elongation, mycelium growth and sporulation (Lunn, 2011). SDHI fungicides specifically inhibit fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II (McKay et al., 2011). The primary biochemical mode of action is the blockage of the TCA cycle at the level of succinate to fumarate oxidation, leading to an inhibition of respiration (Sierotzki et al., 2013). Fluopyram has a broad spectrum of activity against a diverse group of plant pathogens. Its activity includes several pathogens belonging to the Ascomycetes and Deuteromycetes, such as *Botrytis* spp., *Sclerotinia* spp., and *Monilinia* spp. and *Venturia inaequalis* (Veloukas and Karaoglanidis, 2012; Villani et al., 2016).

CHAPTER 2

MATERIALS AND METHODS

The field trials were conducted in Carbondale, IL in 2015 and 2016. The trial was planted on June 22nd in 2015 and May 31st in 2016. In 2015, the plot was 58.5 m by 33.5 m and in 2016 the plot was 192.5 m by 24.4 m. It was a four-row plot, 3.04 m wide by 6.1 m in length with 0.76-m row spacing. Planting density was 8 seeds/ 30.5 cm with a planting depth of 2.54 cm. The field trial was set up as a randomized complete block design with five replications. It had a factorial treatment structure with 4 varieties in 2016 and 7 varieties in 2015 with 3 seed treatment options. Varieties F, G and H were not included in 2016. All soybean varieties were chosen based on relative maturity, ranging from early to late maturity group four. Pearson et al., (1984) found that cultivars that mature later in the season may escape some of the stress associated with high temperatures and low moisture in soil. The variety names were not provided. The varieties selected were resistant to soybean cyst nematode (SCN) Hg Type 0. The first seed treatment will be referred to as the base treatment and contained a mixture of prothioconazole, penflufen, metalaxyl, clothianidin, and *Bacillus firmus* i-1582. The second treatment contained the base treatment plus fluopyram. A third treatment consisted of a non-treated control.

The trial area was inoculated with *M. phaseolina*. In each plot, the two center rows were inoculated with a rate of 4 g of inoculum per 30.5 cm in furrow while planting. The seed and the inoculum were dropped in the furrow at the same time. The trial area was naturally infested; however, inoculum was used to insure each plot had sufficient density of the pathogen. The inoculum was prepared using a protocol described by Wenefrida et al., (1997) with some modifications. Modifications included using white grain sorghum, [*Sorghum bicolor* (L.) Moench] (Janet, 1983) as the inoculum carrier. Sorghum seeds were soaked in a Rubbermaid

37.8-liter container for approximately 17 hours in tap water. The next morning 1,360 g of grain sorghum was placed in aluminum pans (29.8 cm x 23.8 cm x 5.8 cm) with the aluminum lid tightly secured leaving approximately 6 inches left open for ventilation. One of two pieces of heavy duty aluminum foil (71.1 cm long by 11.4 cm wide) are wrapped around each side of the tin allowing some of the aluminum foil to be on the top of the tin. The aluminum tins were sterilized for 15 minutes at 132°C in an Amsco Eagle 3000 Stage 3 autoclave. The whole sterilizing process with dry down and releasing pressure takes a total 35 minutes. Once the tins were autoclaved, the lids were shut completely to minimize contamination. The tins were then stored at room temperature to cool overnight and were autoclaved for a second time the following day. The aluminum tins sat overnight again at room temperature to cool completely before being inoculated. Using a Laminar Flow Hood to reduce contamination, four potato dextrose agar (PDA) petri dishes that contained actively growing *M. phaseolina* mycelium and 600 ml of sterile potato dextrose broth (PDB) were blended using a Waring Commercial Blender. Each tin received 100 ml of the blended broth and agar solution. The aluminum lids were closed on all four sides and a 50.8-cm-long piece of parafilm was wrapped around the lid and side of the tin and the aluminum foil was put back on the tin. The aluminum tins were incubated at room temperature for 7 days and then air dried at room temperature for 3 days. Once the inoculum was dry, it was sieved using a Seedburo Grain Sieve (0.4 cm x 1.9 cm) round steel sieve and put in brown paper sack (38.1 cm x 8.8 cm x 91.4 cm). The inoculum was stored in a cold storage unit, 4.4° c until the trial was planted.

Two weeks after emergence, soil samples were collected to determine the initial levels of soybean cyst nematode (SCN) *Heterodera glycines* (Mengistu et al., 2007). The samples were collected from the 2 inoculated rows from each plot. In each plot, 6-8 cores, 15.2 - 20.3 cm deep

were collected in a zig-zag pattern (Todd, 1993). Each sample was mixed by hand in the Ziploc bag so that there were no clumps to ensure good representative samples. A 100 ml Nalgene polypropylene beaker was filled with 200 ml of tap water and 100 cc of soil was added to the beaker. The soil and water was transferred from the beaker to a 3-liter pitcher. The soil and water was blasted with tap water filling the 3-liter pitcher up 2.54 cm from the top of the pitcher. The soil and water mixture was allowed to settle for 8 seconds then poured over a 707- µm (micron) sieve over 177-µm sieve. The soil and water was blasted again using tap water and let to settle for 8 seconds. The mixture was again poured over a 707-µm sieve over 177-µm sieve. The 707-µm sieve was rinsed with tap water insure all cysts were on the 177-µm sieve. The 707-µm sieve material was discarded, and the 177-µm sieve material was collected into a non-sterile 4-oz polypropylene specimen container with a screw on lid. The cysts were crushed using a drill press with a motorized stirrer and rubber stopper. Tap water was used to rinse the cysts from the specimen container into a small PVC sieve. A 75 µm over a 25-µm sieve was then used to catch all the eggs. The sample was ground until nothing remained and the tap water was clear. The 25 over 75-µm sieve was rinsed with tap water and the eggs were collected on the 75-µm sieve. The eggs were rinsed into the specimen container using tap water and 7 drops of acid fuchsin (3.5 grams acid fuchsin, 250 ml glacial acetic acid and 750 ml distilled water) and tap water was added up to 40ml prior to heating the samples in the microwave for 2 minutes. Acid fuchsin is used so the eggs are easier to see under the microscope since nematodes are translucent and hard to see. Once cooled, each sample is checked to make sure there is 40 ml in the container. For each sample, a 1 ml subsample of the solution is counted. The eggs were counted with a stereomicroscope. Each sample was recorded as egg/ml. To get the total amount of eggs in 40 ml, the number of eggs was multiplied by 40.

Plant Population Assessment

Plant population (stand) was determined at the V4 growth stage in the center two rows. Root samples were collected 30 days after planting and at harvest for colonization of *M. phaseolina.* Six plants per plot were collected using a shovel to ensure removal of the entire root system. The top of the plant was cut off at the soil line and the roots were rinsed using tap water, blotted dry and placed in a brown paper sack to dry in the greenhouse. Once the roots dried, each root was split in half and were rated for charcoal rot symptom severity using an established root rating scale (Mengistu et al., 2007) that ranged from 1-5, 1 = no microsclerotia visible in the tissue to 5 = tissue is darkened due to the high number of microsclerotia visible. Only the harvest roots were rated. The 30-day roots did not have any visible symptoms. The roots were ground by using a Wiley Laboratory Mill root grinder and the root tissue from each sample was placed into polystyrene dilution vials. After each sample the root grinder was cleaned using an air compressor. Colony forming units (CFUs) were determined from the inoculum and root samples. For the inoculum CFUs, 0.5 g of the inoculated sorghum was poured into 100 ml of potato dextrose agar that was amended with 100 mg rifampicin and 1 ml tergitol and poured into the 250 ml Erlenmeyer flask. The sample was swirled, to obtain uniformity and distributed evenly among 10 petri dishes. The plates were incubated at room temperature for 3 days in the dark and then counted for total CFUs per plate. In 2015, there was 1,300 CFU per gram. The CFUs in the root samples were determined in a similar manner to the inoculum. A total of 0.5 g of tissue was weighed in weighing canoes using a Denver Instrument TP-214 Analytical Balance. The sample was placed in a blender with 100 ml of 0.525% NaOCl. In 1-minute intervals, the sample was processed (blend-rest-blend-rest-blend) for a total of 5 minutes. The suspension was poured over a 44-µm sieve and rinsed with tap water. The sample was backwashed into a sterile, 250 ml

Erlenmeyer flask using minimal amounts of distilled water. 100 ml of cooled potato dextrose agar that was amended with 100 mg rifampicin and 1 ml of tergitol was poured into the Erlenmeyer flask. The sample was swirled and distributed among 10 petri dishes. The plates were incubated at room temperature for 5 days in the dark and counted for CFUs.

Root Colonization Assessment

DNA extraction and quantification polymerase chain reaction (qPCR) was completed for assessing root colonization of *M. phaseolina*. The cetyltrimethylammonium bromide (CTAB) method for DNA extraction was used. CTAB is a non-ionic detergent that can precipitate nucleic acids and acidic polysaccharides from low ionic strength solutions (Sambrook et al., 2001). Quantitative PCR is used to measure the quantity of a PCR product (Joshi et al., 2010). It is used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample (Joshi et al., 2010). Absolute quantification is the method that was used for qPCR. This method is based on a standard curve of known quantities. All the unknowns, which are the samples that are being quantified are compared to the standard curve. The root tissue was ground using liquid nitrogen in a sterilized mortar and pestle. For each sample, 0.1 g of tissue was placed into a 1.5 ml sterilized plastic microcentrifuge tube that has a tight-fitting hinged cap. Each sample was placed in a styrofoam container that had ice in it until all the samples were ground. Once all the samples were ground and ready for DNA extraction they were transferred to polypropylene microcentrifuge PCR tube racks to keep the samples upright. In each sample, 800 µl of CTAB buffer was added to each sample and vortexed for three seconds. Before the use of CTAB buffer, 1% polyvinylpyrrolidone (PVP) and 3% beta-mercaptoethanol is added to the buffer. The samples were placed in a 55°C water bath for 3-4 hours. Every 45 minutes the samples were vortexed and then inverted and vortexed at the end of the 4 hours. At the end of the

4 hours 600 µl of a 24:1 ratio (chloroform: isoamyl alcohol) was added, inverted and centrifuged in an Eppendorf Centrifuge 5417C for 5 minutes at 120,000 rpm. The supernatant was transferred to a new set of microcentrifuge tubes and 400 µl of a 24:1 ratio (chloroform: isoamyl alcohol) was added and centrifuged for 5 minutes at 120,000 rpm. The top aqueous layer was transferred to another set of microcentrifuge tubes and 400 µl isopropanol was added to the tube and put in a -20°c freezer overnight. The next day the samples were centrifuged for 10 minutes at 140,000 rpm. The supernatant was discarded leaving the pellet at the bottom of the tube. To clean the pellet 400 µl of 75% ethanol was added to the tube and vortexed. The samples were placed into the centrifuge for 7 minutes at 140,000 rpm. The supernatant was discarded again and 400 µl of ice cold 95% ethanol was added. The samples were centrifuged for 7 minutes at 140,000 rpm. The supernatant was discarded, and the lid of the microcentrifuge was left open to allow the samples to dry. Once the pellet was dry, 50 µl of pure water was added to each tube to help the pellet dissolve. The samples were placed in the -20°C freezer overnight. The microcentrifuge tubes were vortexed and centrifuged for 7 seconds the next morning to ready for DNA quantification.

Root Assessment

DNA quantification was determined for each sample by using a Nanophotometer (ng/µl). The spectrophotometer determined the average concentration of the nucleic acids DNA present in a sample. The ratios of absorbance used were 260/280 and 260/230. Nucleic acids absorb ultraviolet (UV) light due to the heterocyclic rings of the nucleotides. The wavelength of maximum absorption for DNA is 260nm (Anonymous, 2011). The two ratios are indicators of DNA purity. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA (Anonymous, 2009). The ratio 260/230 is used as a secondary measure of nucleic acid purity

(Anonymous, 2009). Before starting, the cell holder was cleaned using TE Buffer. Each sample was measured and quantified separately using 1.5 µl of DNA. To prepare the samples for QPCR, the samples needed to have the same amount of DNA. Using a new set of microcentrifuge tubes, the desired amount of water was added to each and 10µl of DNA from each sample was added to the tube. The samples were vortexed for a second then put in a -40° refrigerator overnight. The next day a few samples were measured using the Nanophotometer to see how much DNA was in the sample before running qPCR. The samples were put on ice to thaw while making the master mix. To prepare for making the Master Mix all the lights were turned off. The probe used was light sensitive and direct light could interfere with the efficiency of the probe. DNA is prone to contamination, and the area was cleaned to prevent any contaminates. The master mix was made right before running the qPCR. The master mix included water (5.92 µl per reaction), 5x GoTaq Flexi Buffer (4 µl per reaction of 10mM), $MgCl_2$ (2.4 µl per 25mM), dNTPs (0.4 µl per reaction of 10mM), F + R primer (1.5 µl per reaction of 10 µM), probe (0.5 µl per reaction of 10µM), GoTaq Flexi polymerase (0.2 per reaction of 5µ/µl) and BSA (0.08 µl per reaction of 10 µg/µl). A well-plate map was designed so the correct DNA sample went into the correct well. Once the master mix was made and the samples were thawed, 15 µl of the master mix and 5 µl of each sample DNA was put into each well of a 96-well plate. Avant Guard low binding barrier tips were used to put each DNA sample into the corresponding well. These sterile tips improved the accuracy of volume dispensing and were ideal for sensitive assays like qPCR. The well plate was put into a frozen PCR-cooler well plate, so the samples were kept cold. The plate was covered with an adhesive PCR plate seal and was made sure the seal was tightly sealed to each individual well. The plate was centrifuged in a miniplate spinner for 30 seconds. After centrifugation the plate was carefully put into the CFX96 Real-Time System, the machine that runs the qPCR. To

check the efficiency of the data a standard curve was used by preparing a dilution series of templates of known concentrations. It was used to calculate the unknown quantity of target DNA in the samples. Standard concentrations of known *M. phaseolina* DNA and 45 µl of water was used. The standards were processed with the samples to evaluate the efficiency of the amplification. There were 6 dilutions, each dilution having 10x more the DNA, the concentrations went from lowest concentration to highest concentration, 0.01, 0.1, 1.0, 10.0, 100.0. Each concentration was replicated twice. Along with the standards, NTC (no template control) was processed with the samples. NTC was the control that does not contain the DNA and can detect contaminations. The Taqman probe was labeled with FAM (fluorescein). FAM was monitored during each PCR cycle providing an amplification plot during the reaction. During the qPCR process there were three steps that were involved, denaturing, annealing and extension. First, the DNA was denatured at high temperatures (95° C) and the double-stranded template was separated into single-strands. During annealing the temperature was lowered and the primers attached to the complementary sequences of DNA making it double-stranded again. Extension was the final step and the temperature was increased and new DNA was made by Taq polymerase which added the bases. The thermal cycle was repeated 50 times. The end result was a quantification cycle (Cq) value that corresponded to each sample giving the amount of quantification of *M. phaseolina* in the sample. The result was an exponential increase in the total number of DNA fragments that included the sequences between the PCR primers (Joshi et al., 2010).

Plant Height and Seed Assessment

In 2016, plant height was taken at the growth stage R5 on four random plants in the center two rows. There was a visible height difference across the trial. A 182.88 cm straight edge

metal ruler was used to measure from the soil line to the top of the plant. In 2015, a seed quality assessment was taken at harvest to evaluate if there were any visible differences between varieties or if the pathogen caused any seed quality damage. The center two rows were harvested and all the seed from each plot was bagged so a subsample could be collected for seed quality rating. The scale was a percentage 0-100, 0 being no visible discoloration or damage of the seed and 100 being the entire subsample was discolored and damaged. Both years, the trial was harvested using a two row Kincaid 8-XP combine. The center two rows were harvested and yield data was collected. The data included test weight, moisture, seeds per pound and yield. In 2015, the trial was harvested on October 19th and in 2016 on October 17th. In 2015, the seed from each plot was saved and seed quality was determined.

The general linear mixed models (GLIMMIX) procedure of SAS was used for all analyses. Fixed effects for the models were variety, treatment, year and all interaction. The experiments were viewed as multiple environments trials. Fisher's Protected least significant difference was used for mean separations at a significance level of $P < 0.05$.

CHAPTER 3

RESULTS

The total rainfall from May to September in 2015 was 65.4 cm and in 2016 was 72.9 cm (Fig 1) and was above the 29-year average of 50.7 cm. In 2015, the months of May and August were below the average rainfall and June, July and September was above the average rainfall. There was more rainfall in 2016 than in 2015. In 2016, only June was below average. For both years, July had the most rainfall. The average air temperature (Fig. 2) for each month was fairly close to the 26-year average for both trial years.

For assessment on *M. phaseolina* colonization, qPCR was determined 30 days after planting (DAP). The quantity of *M. phaseolina* DNA amount ranged from 0.04 ng in variety H to 0.30 in variety C. Variety C contained more of *M. phaseolina* DNA than all the other varieties. All three seed treatment options had similar levels of *M. phaseolina* DNA. The QPCR assessment for the harvest roots at 120 DAP had a higher amount of *M. phaseolina* DNA. Variety F had more *M. phaseolina* DNA than variety G and H but similar to the other varieties. There were differences between seed treatment options, base fungicide had higher *M. phaseolina* DNA than the base treatment with fluopyram but was similar to the non-treated. Another method to assess colonization of the pathogen was assessing the CFU in dried root material. The CFU per gram of root ranged from a low of 2,511 for variety G to a high of 5,487 for variety C. All the other varieties were similar in CFU. There were no differences between seed treatment options for CFU. Root rating assessed the severity of root symptoms and signs of the pathogen. Variety F had a higher root severity than varieties A, B C and H, but was not different from varieties E and G. All three seed treatment options had a similar root rating severity.

The impact of varieties and seed treatment options on plant parameters are shown in Table 2. Visually, in 2015 stand was uniform and in 2016 the stand was inconsistent. Variety B had more plants/plot than variety A and F, but was the similar to variety C, E and G. The seed treatment options had similar plants/plot. Since there were visual height differences between plots, plant height was evaluated in 2016. Varieties F, G and H were not available in 2016. Variety C had the highest height among the varieties, with the other varieties similar in height. Plant height did not differ for the seed treatment options. Varieties A and E had the poorest quality seed and were similar to each other. All other varieties had good seed quality. Seed quality was similar for the base treatment and the base treatment plus fluopyram. Soybean yield differed among varieties but not by seed treatment. Variety F had the lowest yield and averaged 3308.7 kg/ha and variety C had the highest yield and averaged 3671.9 kg/ha. Varieties A, B, C and E were similar and different than F, G and H. All three seed treatment options were similar in yield.

The impact of varieties and seed treatment options on SCN population densities are parameters are shown in Table 3. SCN population densities were determined 2 weeks after planting. There were not any differences across varieties or seed treatment options.

CHAPTER 4

DISCUSSION/CONCLUSION

The environment has been shown to be a major contributing factor in Charcoal rot development (Radwan et al., 2014). The pathogen grows actively, competes for substrate, and predominates in host tissues more effectively than other fungi during dry conditions (Wyllie, 1989). Colonization of the root by the pathogen is higher when plants are subjected to post flowering water stress (Tosi and Zazzerini, 1990; Diourte et al., 1995). The symptoms of the disease are most evident during the reproductive phases of the plant growth (Kendig et al., 2000). The amount of rainfall during the two growing seasons of this study was not conducive for expression of charcoal rot symptoms. There was abundant rainfall throughout both growing seasons, especially during the peak months when the plant was in the reproductive stages.

Formation of microsclerotia is an important survival mechanism for *M. phaseolina* (Baird et al., 2003). Depending on the environmental conditions and association of the microsclerotia with the host residue, microsclerotia can normally survive for 2-15 years (Short et al., 1980; Baird et al., 2003). Unless destroyed by environmental factors or other microorganisms, these structures will continue to germinate and infect host tissues during subsequent growing seasons (Baird et al., 2003). In wet soils, microsclerotia do not survive more than 7-8 weeks and mycelia cannot survive more than 7 days (Hartman et al., 2015). Microsclerotia levels decrease as water levels increase, and soils at 60% moisture-holding capacity are sufficient to keep fungal populations at bay (Dhingra and Sinclair, 1975). While moisture-holding capacity was not measured in the current study, the above average rainfall would have impacted the microsclerotia in this study.

The air temperatures during this study were conducive for the pathogen, but there were periods of cooler weather. In 2015, during the month of July there were 5 consecutive days followed by 4 days that had below the 26-year average temperature. For the month of August, there were 4 days and then 10 consecutive days that had below average temperatures. September started out with temperatures above average and then there was a period of 10 of the 14 days with below average temperatures. In 2016, July started out below the average temperature and then stayed above the average for the rest of the month. August started out above the average and by the middle of the month the temperature went below the average for 8 days. The end of the month was above average temperature. September was above average temperature except a few days at the beginning of the month. The end of the month had below average temperature for 5 days.

Diseased plants may wilt and prematurely die with senesced leaves remaining attached to the petioles (Mengistu et al., 2009). Aboveground visual symptoms of the disease were not observed throughout the whole growing season. This could be due to the amount of atypical soil moisture and the low temperatures in the months of July, August and September.

Molecular tools such as real time PCR are one of the ways used in pathogen detection and quantification (Azarmanesh, 2013). Babu et al., (2007) reported the first development of specific primers and probes for the identification and detection of *M. phaseolina*. Later which qPCR assays with greater specificity and sensitivity detect *M. phaseolina* was by Babu et al., (2011). Quantitative real-time PCR based assays have advantages of speed, accuracy and sensitivity over other detection techniques (Gachon et al., 2004; Schaad and Frederick, 2002; Schena et al., 2004; Wong and Medrano, 2005). In this study, the qPCR data revealed that there was minimal amount of *M. phaseolina* DNA in root tissue at 30 DAP. This could be the reason

there was not visible discoloration in the root samples when the roots were rated at 30 DAP. The 120 DAP qPCR data revealed there was more *M. phaseolina* DNA present in the root tissue. This may be due to *M. phaseolina* growing rapidly during the R5, R6 and R7 plant growth stages and resulted in more DNA. This agrees with the work of Gupta et al., (2012) when the pathogen was more prevalent post flowering stage (Gupta et al., 2012).

Significant efforts have been made to identify charcoal rot resistance in soybean, but no claims have been made for soybean charcoal rot resistance in commercial varieties (Bellaloui et al., 2008). This is in contrast with a report that was based on seed yields and the levels of lower stem and taproot colonization by *M. phaseolina*; four soybean cultivars, Asgrow 4715, DeltaPineland 3478, Hamilton, and Jackson II were rated moderately resistant to *M. phaseolina* (Smith and Carvil, 1997). Results from a study by Smith and Carvil, (1997), demonstrated that field screening soybean cultivars for resistance to *M. phaseolina* is a reliable though time intensive process. Consistent with other research results, Smith and Carvil, (1997) observed that all soybean cultivars may become infected by *M. phaseolina*. Resistance factors to *M. phaseolina* do not protect plants against infection, but more likely restrict the growth rate of the fungus within plant tissue (Smith and Carvil, 1997).

Fungicide treatment of soybean seed can help manage seedling damping off and seed rot problems caused by fungi (Heatherly and Elmore, 2004). Several seed treatments are available for commercial use, *Pythium* spp., *Phytophthora sojae*, *Rhizoctonia* spp., and *Fusarium* spp. are the most common pathogens associated with reduced soybean germination and emergence and subsequent stand failures (Heatherly and Elmore, 2004). There are currently no fungicide seed treatments to manage charcoal rot in soybean. For this study, seed treatments did not affect root colonization at 30 DAP, root rating, plants/plot, height, seed quality or soybean yield. The only

differences in regards to seed treatment was in the qPCR 120 DAP. The base treatment had more *M. phaseolina* DNA than the other two treatments. It is possible that the seed treatments controlled other root pathogens, thereby allowing for greater concentrations of *M. phaseolina*. Mengistu et al. (2015) reported that seed treatments may be helpful if soybean seeds are infected with *M. phaseolina*, but there was no information on specific active ingredients effective against this pathogen. The fungicide fluopyram was registered in December 2014 for the treatment of soybean seed to manage Sudden Death Syndrome (SDS) (Kandel et al., 2016). There are reports of the use of seed treatment and its impact on yield for other seed borne pathogens that have shown positive responses. In this study, fluopyram and the other seed treatments had little impact on *M. phaseolina*.

Quantification of microsclerotia in stem and root tissue as CFU can be useful as a pathogen assessment method (Smith and Carvil, 1997). One problem with this method is the variability within a single sample and also among years. The same amount of root tissue was used when preparing for CFU during both years. Another method that was used to assess the disease was to rate the lower stem and tap root. This method also showed variability within a single plot. The established root rating scale was used but, was adjusted for this study due to the large number of microsclerotia found in the root tissue. The root rating scale was on a scale from 1 to 5 just like the established scale, 1 being no microsclerotia visible to 5 being the tissue was darkened due to the high numbers of microsclerotia. It was adjusted for this study in that each number there was more microsclerotia visible than in the established scale. For both years, within a single plot the ratings ranged from 1-5.

One of the symptoms of charcoal rot includes stunted growth. In sunflower, charcoal rot can reduce stem height (Hoes, 1985; Kolte, 1985). To evaluate if visual height differences were

caused by *M. phaseolina* in this study plant height was recorded, and the results indicated that the visible height differences observed across the trial area were due differences in varieties and did not correspond to the three seed treatment options.

A visual rating of seed quality was performed after harvest. A subsample was taken from each plot and was visually assessed. Overall, there was not much damage to the seed. In Gangopadhyay et al., (1970) four varieties were tested and found that infected seeds have indefinite black spots and blemishes on the seed coat. Many of the seeds harvested from the inoculated plants were blemished by the presence of black, nondescript spots whereas seed from noninoculated plants were blemish-free (Gangopadhyay et al., 1970). Their data suggests that under severe field infestations of *M. phaseolina*, a considerable percentage of seed can be infected; that the pathogen is capable of surviving for extended periods in the seed coat (Gangopadhyay et al., 1970).

Yield losses due to charcoal rot can vary among years. In sunflower, losses from charcoal rot can reach 60 to 90% if the conditions are favorable for infection (Khan, 2007). *M. phaseolina* can suppress yield loss depending on the timing and severity of the disease. Yield loss assessments of charcoal rot have been largely based on anecdotal evidence, so replicated variety trials are needed in areas where the disease is a recurrent problem (Smith and Carvil, 1997). The amount of yield loss attributed to charcoal rot cannot be determined when there is drought stress associated with non-irrigated treatments (Mengistu et al., 2011). This is in agreement with Bowen and Schapaugh, who reported no association between charcoal rot severity and yield in a non-irrigated environment (Bowen and Schapaugh, 1989). In this study, differences in soybean yield were due to genetic differences of the varieties.

Charcoal rot severity, which is frequently related to drought stress (Meyer et al., 1974; Pearson et al., 1984), may be enhanced in the presence of the *H. glycines* (Radwan et al., 2014). Todd et al., (1987) results indicated that *H. glycines* infection can increase colonization of soybean roots by *M. phaseolina* which increase losses due to charcoal rot. In another study, Francl et al., (1988) found no interaction between *M. phaseolina* and *H. glycines* in a field where both organisms were present. *H. glycines* did not have an impact on variety or treatment for this study. This could be due to the timing the samples were taken. The focus for SCN for this study was to document the presence and also the distribution in the study.

It is difficult to study charcoal rot since the growing conditions can change from year to year. The amount of rainfall and the periods of cooler weather may have slowed down colonization. The data from this study indicated that applying a seed treatment did not influence *M. phaseolina* colonization. Colonization of *M. phaseolina* was different among the different varieties. Seed treatment did not influence qPCR, CFU and root rating severity. Plant parameters such as stand, plant height, seed quality and yield did not differ across the three seed treatment options. To further seed treatment and any other potential management options, a study needs to be conducted in an environment that is more conducive for charcoal rot.

CHAPTER 5

RECOMMENDATION

To manage the soil borne pathogen, *Macrophomina phaseolina*, current seed treatments are not recommended. However, seed treatments are a good option for other soilborne pathogens. Choosing a resistant variety that is resistant to the prevalent diseases in the area is always something to take into consideration when deciding what seed variety to plant. For *M. phaseolina* that is not an option. Currently the management options to control the pathogen is planting non-host crop to decrease the population in the field, reducing plant stress by planting a lower population or if feasible irrigation. Planting varieties that are later maturity groups may escape some of the stresses of the pathogen by flowering later in the season when the temperatures are lower and there is less rainfall.

EXHIBITS

Table 1. Quantification of Macrophomina phaseolina colonization in soybean roots and symptoms severity as influenced by variety and seed treatment.

Factor	QPCR (ng) 30 DAP	QPCR (ng) 120 DAP	CFU /0.5 gram root 120 DAP	Root Rating Severity 120 DAP (1-5 scale)
Variety				
A	0.08 b	0.8 ab	3,401 ab	2.1 c
B	0.09 b	0.7 ab	3,447 ab	2.5 bc
C	0.30 a	1.0 ab	5,487 a	2.6 b
E	0.07 b	1.4 ab	5,081 ab	2.8 ab
F	0.07 b	2.4 a	3,787 ab	3.2 a
G	0.05 b	0.3 b	2,511 b	2.8 ab
H	0.04 b	0.5 b	3,364 ab	2.3 bc
Treatment				
Base Treatment	0.07 a	1.7 a	3,688 a	2.7 a
Base Treatment plus Fluopyram	0.06 a	0.7 b	3,886 a	2.5 a
Non-treated	0.17 a	0.6 ab	4,031 a	2.8 a

[1]*Within each factor and parameters, means with similar letters are not different according to Fishers protected LSD ($P \leq 0.05$)*

Table 2. Soybean plants/ plot, plant height, seed quality, and yield as influenced by variety and seed treatment.

Factor	Plants/plot	Plant Height R5 (cm)	Seed Quality Scale 0-100%	Soybean Yield kg/ha
Variety				
A	258.9 bc	111.0 b	4.9 a	3611.4 a
B	285.7 a	109.5 b	1.4 b	3665.2 a
C	281.1 ab	116.6 a	1.5 b	3671.9 a
E	270.2 abc	109.5 b	4.8 a	3618.1 a
F	255.1 bc	.	1.5 b	3308.7 c
G	266.3 abc	.	0.7 b	3463.4 b
H	250.1 c	.	0.6 b	3396.2 bc
Treatment				
Base Treatment	269.0 a	111.5 a	2.8 a	3530.7 a
Base Treatment plus Fluopyram	264.8 a	113.0 a	1.6 a	3537.4 a
Non-treated	266.5 a	110.2 a	.	3530.7 a

[1]Within each factor and parameters, means with similar letters are not different according to Fisher's protected LSD ($P \leq 0.05$)

Table 3. SCN as influenced by variety and seed treatment

Factor	SCN/100cc 2 WAP
Variety	
A	716.1 a
B	400.5 a
C	437.1 a
E	569.8 a
F	392.6 a
G	384.6 a
H	357.9 a
Treatment	
Base Treatment	475.0 a
Base Treatment plus Fluopyram	387.4 a
Non-treated	534.2 a

Means with similar letters are not different according to Fishers protect LSD ($P \leq 0.05$)

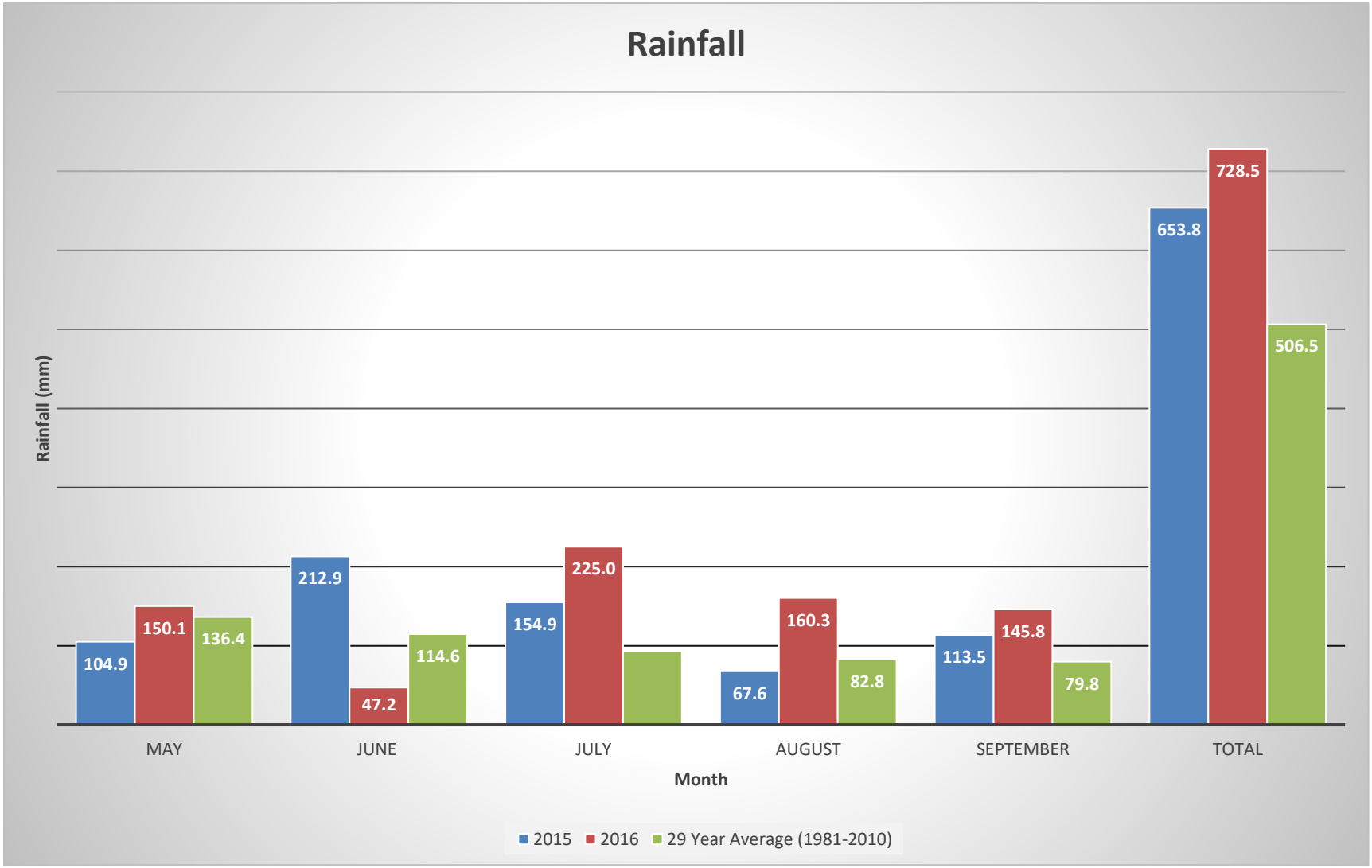

Figure 1: Total rainfall by month and the 29-year average for that month in 2015 and 2016 in Carbondale, IL

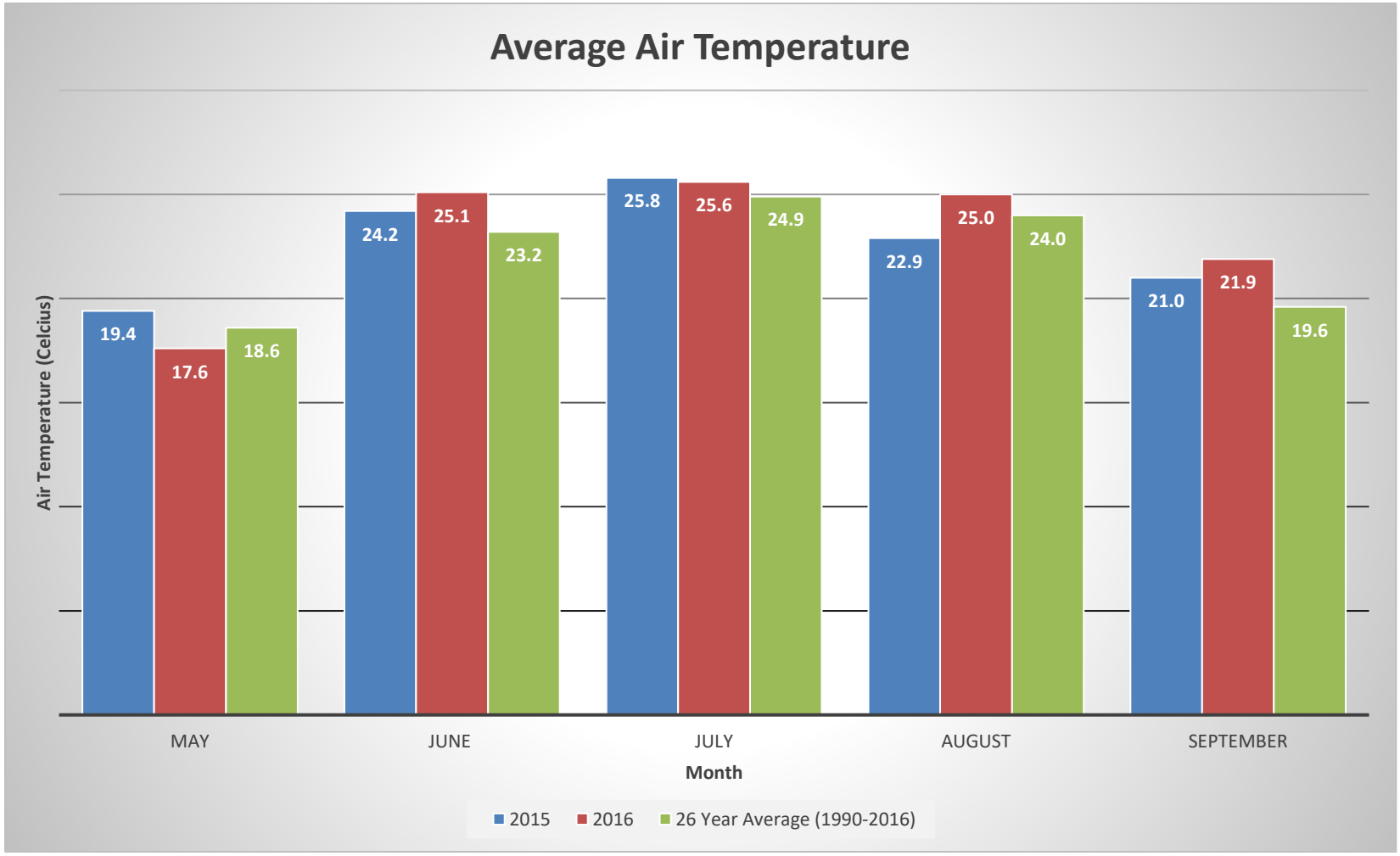

Figure 2: Average air temperature by month and the 25-year average for that month in 2015 and 2016 in Carbondale, IL

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VITA

Graduate School
Southern Illinois University

Annie C. Padgett

ackorando@gmail.com

Southern Illinois University Carbondale
Bachelor of Science, Crop, Soil and Environmental Management, May 2015

Thesis Title: THE EFFECTS OF FLUOPYRAM ON *MACROPHOMINA PHASEOLINA* AND CHARCOAL ROT OF SOYBEAN

Major Professor: Jason P. Bond